

DNA and hemoglobin alkylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in F344 rats

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Alkylation of DNA and hemoglobin was compared in male F344 rats given a single s.c. injection of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), or its major metabolite formed by carbonyl reduction, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). In hepatic DNA, levels of 7-methylguanine and *O*⁶-methylguanine formed from NNK 1–48 h after treatment were similar to those formed from NNAL. In nasal mucosa and lung DNA, levels of 7-methylguanine and *O*⁶-methylguanine were somewhat higher after treatment with NNK than with NNAL. Acid hydrolysis of hepatic DNA, isolated from rats treated with either [5-³H]NNK or [5-³H]NNAL, gave 180 ± 48 or 120 ± 23 $\mu\text{mol/mol}$ guanine, respectively, of 4-hydroxy-1-(3-pyridyl)-1-butanone. Basic hydrolysis of globin isolated from rats treated with either [5-³H]NNK or [5-³H]NNAL gave 4.1 ± 0.7 or 2.0 ± 0.1 pmol/mg, respectively of 4-hydroxy-1-(3-pyridyl)-1-butanone. These results indicate that NNAL is not a detoxification product of NNK, since treatment of rats with NNAL results in modifications of DNA which are qualitatively and quantitatively similar to those observed upon treatment with NNK. Alkylation of DNA and globin by NNAL may result mainly from its metabolic reconversion to NNK.

Introduction

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*), a tobacco-specific nitrosamine produced by nitrosation of nicotine, is an important carcinogen found in tobacco and tobacco smoke (1,2). It is highly tumorigenic in rats, mice and hamsters. It induces both local and systemic tumors, with particular organospecificity for lung. In F344 rats, s.c. injection of NNK produces tumors in the lung, nasal cavity, and liver (3–5).

Pharmacokinetic studies of NNK in rats, mice, hamsters and baboons have shown that it is rapidly converted to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by reduction of its carbonyl group (6,7). Cultured human and laboratory animal tissues as well as subcellular fractions can also metabolize NNK extensively to NNAL (8–10). The metabolic activation of NNK to intermediates which methylate and 4-(3-pyridyl)-4-oxobutylate DNA is initiated mainly by hydroxylation of the carbons α to the nitrosamino group as indicated in Figure 1 (1,2,11). Metabolism of NNK also produces intermediates which 4-(3-pyridyl)-4-oxobutylate hemoglobin (12). The role of NNAL in these processes was not known. Therefore we compared the DNA and globin binding of NNK and NNAL in the F344 rat.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; 7-mG, 7-methylguanine; *O*⁶-mG, *O*⁶-methylguanine; HPLC, high performance liquid chromatography.

Materials and methods

Chemicals

NNK and NNAL were synthesized (8,13,14). [5-³H]NNK (sp. act., 912 mCi/mmol; purity, 98%) and [5-³H]NNAL (sp. act., 934 mCi/mmol; purity, 97%), labeled with tritium at the 5-position of the pyridine ring, were obtained from Chemsyn Science Laboratories, Lenexa, KS. Their purities were confirmed by HPLC. They were not cross-contaminated. They were diluted as necessary with unlabeled NNK or NNAL. Standard metabolites of NNK or NNAL were synthesized (8,15,16). Trioctanoin was obtained from Eastman Kodak Company (Rochester, NY) and was redistilled before use.

Animals

Male F344 rats weighing 220–250 g were obtained from Charles River Breeding Laboratories, Kingston, NY. They were housed under standard conditions (5). They were given s.c. injections of the appropriate compound in 1 ml of trioctanoin and were killed by decapitation under anesthesia.

DNA methylation experiments

In experiment 1, groups of three rats were treated with NNAL (81.5 mg/kg, 0.39 mmol/kg) and killed 1 h, 4 h, 12 h, 24 h, 36 h, and 48 h later. This protocol was identical to one previously used for NNK, as described in reference 5. DNA was isolated from the liver and lungs of each rat and from the pooled nasal mucosa of three rats, as described previously (5). In experiment 2, groups of three rats were treated with NNAL (0.39 mmol/kg) or NNK (81 mg/kg, 0.39 mmol/kg) and killed 12 h or 36 h later for DNA isolation as above.

In Figure 2, the 12-h and 36-h NNAL points are the means \pm SD of values for six rats from experiments 1 and 2. The 12 h and 26 h NNK points are the means \pm SD of values for six rats, three from reference 5 and three from experiment 2. The other NNAL and NNK points are means \pm SD of values for three rats, from experiment 1 and reference 5, respectively.

In Figure 3, the 12-h and 36-h points are the means of two pools of three nasal mucosa DNA samples from rats treated with NNAL (experiments 1 and 2) or NNK (reference 5 and experiment 2). The average deviation from the mean was 24%. The other points are from pooled nasal mucosa DNA of three rats treated with NNAL (experiment 1) or NNK (5).

The data in Table I are from experiment 2.

DNA and hemoglobin 4-(3-pyridyl)-4-oxobutylate experiments

Two groups of three rats were treated with either [5-³H]NNAL (0.39 mmol/kg; 17.5 mCi/mmol) or [5-³H]NNK (0.39 mmol/kg; 12.7 mCi/mmol). After 12 h, blood was collected by cardiac puncture, and globin was isolated as described previously (12). The animals were killed and DNA was isolated from liver, lung, and nasal mucosa, as described above.

Analysis of DNA, globin, and blood

Methylation of DNA was determined by HPLC-fluorescence (5,17). Levels of Compound 6 released upon acid hydrolysis (0.8 N HCl, 80°C, 6 h) of DNA were determined by reverse-phase HPLC with a radioactive flow detector, as described previously (11). The identity of Compound 6 was confirmed by normal phase HPLC (11).

Hemoglobin was isolated, extensively dialyzed and treated with acidic acetone to precipitate globin. The globin was washed three times with acetone and then sonically dispersed in 0.1 N NaOH for 1 h at room temperature. The resulting mixture was analyzed as described (11).

Blood from one rat treated with [5-³H]NNAL was centrifuged to remove red blood cells. The plasma was passed through a Clin-Elut column (Analytichem International, Harbor City, CA) with elution by ethyl acetate. The ethyl acetate extracts were concentrated to dryness, redissolved in 0.1 N HCl and, after neutralization, analyzed by reverse-phase HPLC (11).

Results

Levels of 7-methylguanine (7-mG) and *O*⁶-methylguanine (*O*⁶-mG) in liver and nasal mucosa DNA were determined, 1–48 h after a single s.c. injection of either NNK or NNAL. The results are illustrated in Figures 2 and 3. In liver, the levels

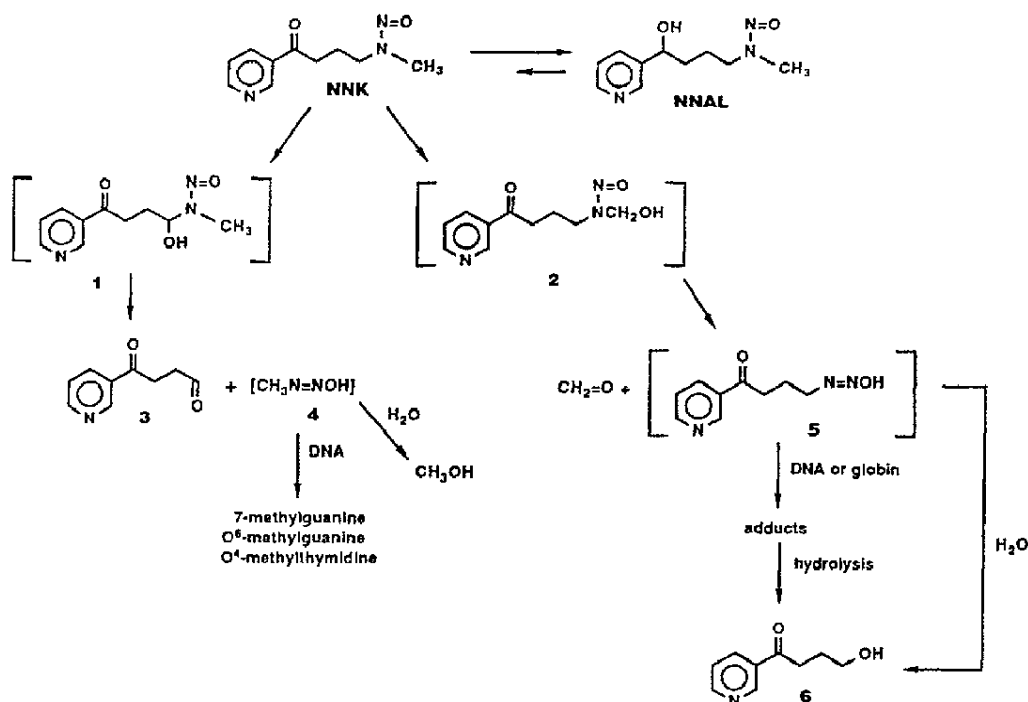


Fig. 1. Metabolic α -hydroxylation of NNK leading to alkylation of DNA and globin.

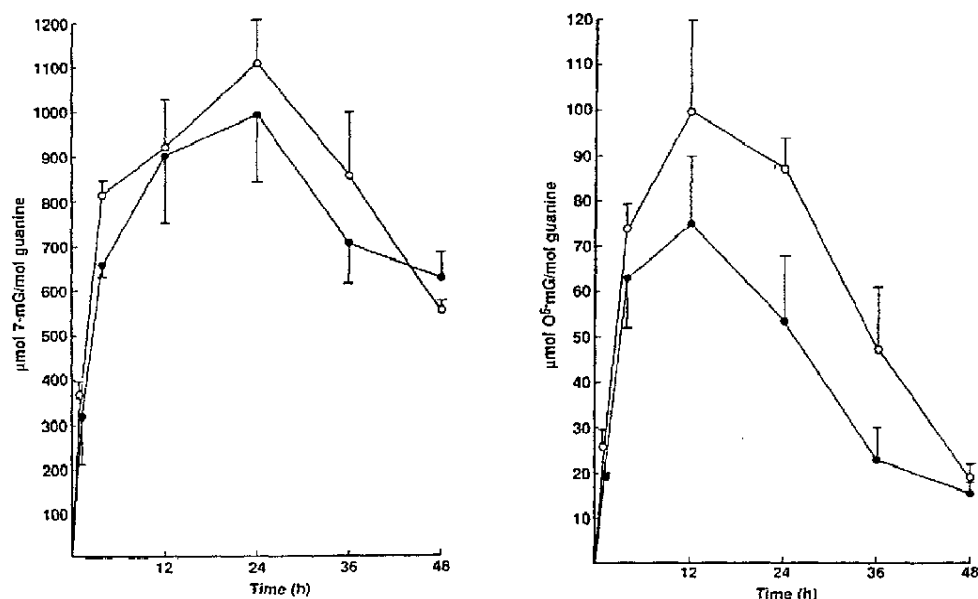


Fig. 2. Levels of 7-mG and O⁶-mG in rat hepatic DNA after s.c. injection of 0.39 mmol/kg of NNK (○—○) or NNAL (●—●). Points are means of values from 3 or 6 rats, obtained as described in Materials and methods.

of 7-mG and O⁶-mG formed from NNK were similar to those formed from NNAL. In nasal mucosa, levels of 7-mG and O⁶-mG produced from NNK were generally somewhat higher than from NNAL.

As observed previously, DNA methylation in lung was lower than in nasal mucosa or liver (5). Methylation was compared 12 h and 36 h after s.c. injection of NNK or NNAL. The results shown in Table I indicate that higher levels of 7-mG and O⁶-mG

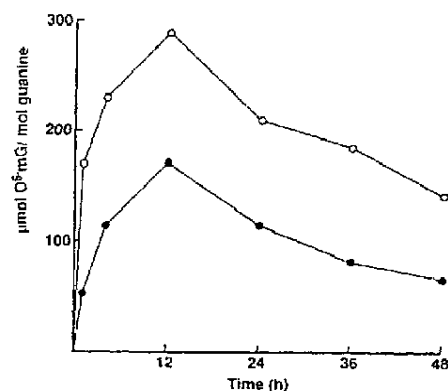
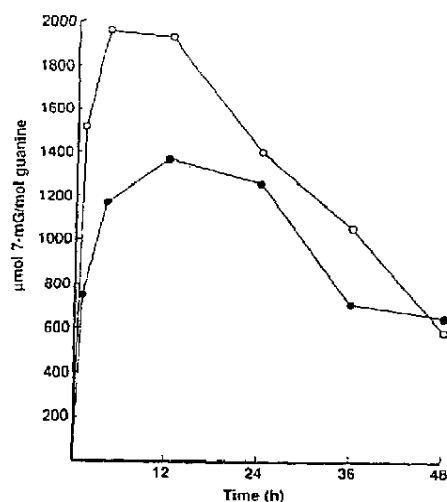


Fig. 3. Levels of 7-mG and O⁶-mG in rat nasal mucosa DNA after s.c. injection of 0.39 mmol/kg of NNK (○—○) or NNAL (●—●). Points are from pooled nasal mucosa DNA samples, obtained as described in Materials and methods.

were formed from NNK than from NNAL.

HPLC analysis of acid hydrolysates of hepatic DNA from rats given s.c. injections of [5-³H]NNAL, and killed 12 h later, gave chromatograms such as that illustrated in Figure 4. A major peak eluting at 52.5 min., which accounted for ~44% of the radioactivity present in the DNA, was identified as 4-hydroxy-1-(3-pyridyl)-1-butanone (Compound 6 of Figure 1) by cochromatography with a standard under reverse phase and normal phase HPLC conditions. Similar chromatograms were obtained upon analysis of acid hydrolysates of hepatic DNA from [5-³H]NNK treated rats (Figure 4), in agreement with previous results (11). Levels of Compound 6 released from hepatic DNA of [5-³H]NNK and [5-³H]NNAL treated rats were 180 ± 48 and 120 ± 23 μmol/mol guanine (mean ± SD), respectively, giving an NNK/NNAL ratio of 1.5. The peak eluting at 47 min has not been identified. No radioactive peaks above background were detected in acid hydrolysates of DNA isolated from nasal mucosa or lung of these rats.

The release of Compound 6 from DNA upon strong acid hydrolysis is believed to result from 4-(3-pyridyl)-4-oxobutylation of DNA via Compound 5 of Figure 1. The ratios of hepatic DNA methylation, as measured by 7-mG, to 4-(3-pyridyl)-4-oxobutylation, as measured by Compound 6, were 5.1 for NNK and 7.6 for NNAL.

Mild basic hydrolysis of globin, isolated from rats 12 h after s.c. injection of [5-³H]NNAL, followed by HPLC analysis of the hydrolysate gave a chromatogram in which the only major radioactive peak eluted at 52.5 min. This peak was chromatographically indistinguishable from Compound 6 under reverse-phase and normal phase HPLC conditions. As reported previously, Compound 6 was also released upon basic hydrolysis of globin isolated from rats treated with [5-³H]NNK (12). The amounts of Compound 6 released from globin were 4.1 ± 0.7 pmol/mg globin and 2.0 ± 0.1 pmol/mg globin from the rats treated with [5-³H]NNK and [5-³H]NNAL, respectively, giving an NNK/NNAL ratio of 2.1. Compound 6 accounted for 6% and 28% of the radioactivity in globin from the rats treated with [5-³H]NNK and [5-³H]NNAL.

The chromatograms obtained upon basic hydrolysis of the

Table I. Levels of 7-mG and O⁶-mG in lung DNA of F344 rats following s.c. injection of NNK or NNAL^a

Compound injected	Survival interval (h)	DNA methylation	
		(μmol/mol guanine) 7-mG	O ⁶ -mG
NNK	12	320 ± 160	18 ± 9
	36	70 ± 9	8 ± 1
NNAL	12	99 ± 7	7 ± 2
	36	38 ± 11	3 ± 1

^aF344 rats were given a single s.c. injection of 0.39 mmol/kg of NNAL or NNK and were killed 12 h or 36 h later. Values are means ± SD for 3 rats.

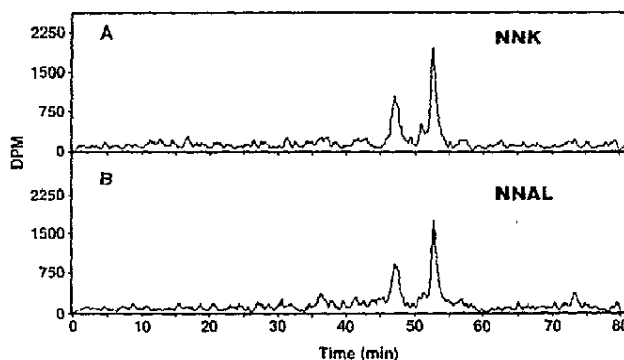


Fig. 4. Chromatograms obtained upon HPLC analysis of acid hydrolysates of hepatic DNA isolated from F344 rats 12 h after s.c. injection of 0.39 mmol/kg [5-³H]NNK or [5-³H]NNAL. The peak eluting at 52.5 min is Compound 6 of Figure 1.

globin from the rats treated with [5-³H]NNK were more complex than those obtained from the globin of the [5-³H]NNAL treated rats, or than those seen in our previous

studies (12), which were carried out at much lower doses (up to 8 μ mol/kg). One of the major peaks other than Compound 6 was identified as NNK, by reduction to NNAL with NaBH₄, and by normal phase HPLC.

The blood of a rat treated with [5-³H]NNAL contained 5.8 and 0.55 nmol/ml of [5-³H]NNAL and [5-³H]NNK, respectively, 12 h after treatment.

Discussion

One of the main goals of this study was to evaluate the potential role of NNAL as a product of metabolic activation or detoxification of NNK. This question is important because NNK is extensively metabolized to NNAL in laboratory animals and in human tissues (6–10). The results indicate that NNAL is not a detoxification product of NNK, since treatment of rats with NNAL results in modifications of DNA which are qualitatively and quantitatively similar to those observed upon treatment with NNK. There has only been one bioassay of NNAL reported. It induced 26 lung tumors per A/J mouse, compared to 38 induced by NNK (9). In F344 rats, NNK and NNAL have similar carcinogenic activities (D. Hoffmann, A. Rivenson and S.S. Hecht, unpublished data). These results appear to be consistent with those obtained in the present study.

The chromatograms obtained upon acid hydrolysis of hepatic DNA from rats treated with [5-³H]NNK or [5-³H]NNAL were similar. The keto alcohol, Compound 6, was the major radioactive peak. The release of Compound 6 from DNA isolated from rats treated with [5-³H]NNAL is consistent with metabolism of NNAL to NNK. α -hydroxylation of NNK, and alkylation of DNA by Compound 5, as illustrated in Figure 1. However, oxidation of a DNA adduct formed from NNAL cannot be excluded. Metabolism of NNAL to NNK, followed by α -hydroxylation, would also account for the release of Compound 6 from globin as observed after injection of either [5-³H]NNAL or [5-³H]NNK. The detection of [5-³H]NNK in blood, after treatment with [5-³H]NNAL, is consistent with this hypothesis and with previous pharmacokinetic studies (6,7). These results, taken together with the similar patterns of hepatic DNA methylation following treatment with either NNK or NNAL,

indicate that most of the hepatic DNA methylation observed after injection of NNAL was due to its conversion to NNK. However, some of the methylation may have resulted from direct α -hydroxylation of NNAL. Metabolism studies have indicated that this process occurs in cultured mouse lung, rat nasal mucosa, and in human tissues (9,10,18). These studies have suggested however that NNAL is a poorer substrate for α -hydroxylation than is NNK.

The position of the NNK–NNAL equilibrium appears to be important in controlling DNA alkylation and perhaps carcinogenesis by NNK. NNK is rapidly converted to NNAL in the F344 rat. The biological half-lives of NNK and NNAL are 25 min and 184 min, respectively (6,7). NNAL is thus a major transport form of NNK. Depending on the route of administration of NNK, tissues may be exposed predominantly to NNK or NNAL. The susceptibility of each tissue or cell type to DNA damage would depend in part upon its ability to oxidize NNAL back to NNK. The relatively lower levels of DNA methylation observed in nasal mucosa and lung after injection of NNAL, compared to NNK, suggest that, at the doses used in this study, either these tissues cannot convert NNAL to NNK as efficiently as liver, and/or that they have a lower capacity to carry out its α -hydroxylation, as indicated in an earlier study with cultured rat nasal mucosa (18). Further experiments are required to assess the position of the

NNK–NNAL equilibrium in various tissues and cell types to more fully evaluate the role of NNAL in carcinogenesis by NNK.

Acknowledgements

We thank Shantu Amin and George Balanikas for synthesizing NNK and NNAL. This study was supported by Grant No. 44377 from the National Cancer Institute. This is paper No. 117 in the series 'A Study of Chemical Carcinogenesis.'

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Received on March 11, 1988; revised on May 20, 1988; accepted on May 24, 1988